Int. J. Peptide Protein Res. 13, 1979, 218–222
Published by Munksgaard, Copenhagen, Denmark

MALEYLATION AND PH-DEPENDENCE OF *STREPTOMYCES GRISEUS* PROTEASE B

SHULAMIT SHINAR and ARIEH GERTLER

Department of Agricultural Biochemistry, Faculty of Agriculture, The Hebrew University of Jerusalem, Rehovot, Israel

Received 22 May, accepted for publication 29 June 1978

The enzymatic activity of Streptomyces griseus *protease B (SGPB) was measured over pH range 8.4–11.5 using a specific new, chromophoric substrate* N-*succinyl-glycyl-glycyl-*L-*phenylalanine* p-*nitroanilide. It was found that the activity is dependent on ionization of a single group with apparent pK = 10.84, possibly lysine-125. Maleylation of the ε-amino group of this lysine was linearily associated with the loss of enzymatic activity.*

It is therefore suggested that the electrostatic interaction between the side chain of lysine-125 and the α-carboxyl group of the C-*terminal tyrosine is crucial to the active conformation of the enzyme. In contrast the maleylation of the α-amino group of the* N-*terminal isoleucine was rapid but could not be correlated to the loss of activity.*

Key words: chemical modification; maleylation; pH dependence of activity; *Streptomyces griseus* protease B.

Streptomyces griseus protease B (SGPB) is the major serine protease found in the commercial preparation known as Pronase® (Jurasek *et al.*, 1971). The enzyme has been studied rather intensively in recent years, including its purification and characterization (Wahlby, 1968; Narahashi & Fukumaka, 1969; Trop & Birk, 1970; Gertler & Trop, 1971; Jurasek *et al.*, 1971; Awad *et al.*, 1972; Siegel & Awad, 1973), primary (Jurasek *et al.*, 1974) and tertiary (Delbaere *et al.*, 1975) structure, specificity (Narahashi & Yoda, 1973), specific inhibition and mapping of its binding site (Gertler, 1974; Gertler *et al.*, 1977). SGPB consists of a single polypeptide chain composed of 184 amino acids and contains only one lysine residue (Jurasek *et al.*, 1974) that forms an electrostatic bond with α-carboxyl group of *C*-terminal tyrosine (Delbaere *et al.*, 1975).

In our previous work (Gertler, 1974) we reported that inhibition of the enzyme by acetyl-L-leucyl-L-phenylalanine chloromethyl ketone was dependent on two ionizable groups with apparent pK's of 6.5 and 9.5. Chemical modifications (Siegel & Awad, 1973) and tertiary structural analysis (Delbaere *et al.*, 1975) revealed that, in contrast to mammalian pancreatic serine proteases, the ionization of *N*-terminal amino group is most likely irrelevant to the enzymatic activity of SGPB.

Therefore the aim of the present work was to clarify whether the hydrolysis of specific nitroanilide substrates at alkaline pH is pH dependent and to identify the group(s) that affect the enzyme activity. We found that the enzymatic activity at alkaline pH is dependent on the ionization of a group with apparent pK

Abbrevations used: SGPB, *Streptomyces griseus* protease B; Suc, *N*-succinyl; Nan, *p*-nitroanilide.

0367–8377/79/020218–05 $02.00/0

of 10.84. By using maleylation as a specific means for modifying amino groups (Butler *et al.*, 1969), we also found that the loss of enzymatic activity was directly correlated with the maleylation of the ε-amino group of the only lysine of SGPB. Maleylation was preferred to other means of primary amino group modification since it is very specific, results in covalent modification that remains stable above pH 4–5 and may introduce a drastic change in protein structure due to an extension of the side chain and replacement of positive by negative charge (Butler *et al.*, 1969).

EXPERIMENTAL PROCEDURES

Materials

Streptomyces griseus protease B (SGPB) was isolated from Pronase® (California Research Corporation) by ion exchange chromatography on CM-Sephadex as described by Jurasek *et al.* (1971). The enzyme was electrophoretically homogeneous, and amino terminal sequence analysis indicated that only a single polypeptide chain was present. Active site titration using *p*-nitrophenyl-*p*'-guanidino benzoate (Cyclo Chemical Co.) at a final concentration of 0.1 mM (Gertler, unpublished data) revealed only 0.68 active sites per molecule of the enzyme. SGPB concentration was determined from its absorbance at 280 mm, using $\epsilon_{280} = 25400\,M^{-1}\,cm^{-1}$. This extinction coefficient was calculated from the amino acid composition, using norleucine as an internal standard. *N*-Succinyl-glycyl-glycyl-L-phenylalanine *p*-nitroanilide ($SucGly_2PheNan$), a new chromophoric substrate of SGPB, was a gift of Dr. S. Blumberg, and *N-tert*-butoxycarbonyl-glycyl-L-leucyl-L-phenylalanine chloromethyl ketone, a highly specific irreversible inhibitor of SGPB (Gertler, 1974), was obtained from Dr. K. Kurachi.

Effect of pH on the activity of SGPB

The activity of SGPB in the pH range of 8.4–11.5 was determined in three buffer systems: 0.1 M glycine-NaOH (pH 8.4–10.4), 0.1 M salicylate–0.15 N NaOH adjusted to the desired pH with 0.1 M glycine-NaOH buffer pH 9.0 (pH 9.8–11.5) and the universal buffer of Britton and Robinson (McKenzie, 1969), (pH 8.8–11.5).

The reaction rates at 30° were estimated by measuring the change in absorbance at 410 nm, following the addition of enzyme to 1 ml of 0.5 mM solution of $SucGly_2PheNan$ in the appropriate buffer.

Maleylation of SGPB

The reaction was carried out at 0°, in 0.1 M tris-HCl buffer, pH 9.2, using an Titrator II-equipped Radiometer pH meter 26 with 1 N NaOH as a titrant. The enzyme concentration was 3 mg/ml in a reaction mixture volume of 2–5 ml. Maleic anhydride (4 M, freshly prepared in dioxane) was added at 5-μl intervals. Residual enzymatic activity was determined in aliquots withdrawn after each addition of maleic anhydride, using the spectrophotometric assay at pH 8.0. Larger samples for the determination of the extent of maleylation were withdrawn simultaneously.

Determination of maleylated lysine

To each 0.3-ml aliquot sample withdrawn at each stage of modification, 0.6 ml of 5% 2,4-dinitrofluorobenzene in ethanol and 12 mg of $NaHCO_3$ were added, and the samples were shaken in the dark at room temperature. After 3 h, the excess reagent was removed by extraction with ether. The sample was acidified with a few drops of 2 M HCl, reextracted with ether, taken to dryness and hydrolyzed in 1 ml constant boiling HCl at 110° for 22 h. The amino acid analysis was performed on an LKB 3201 amino acid analyzer by a single column method. Because ε-amino maleylated lysine residues do not react with 2,4-dinitrofluorobenzene, and because the maleyl group is completely removed by hydrolysis, the amount of lysine detected by amino acid analysis is equal to the amount of maleylated lysine originally present. The quantity of SGPB in each sample was estimated from the amino acid analysis, based on its amino acid composition.

Determination of the maleylated N*-terminal isoleucine residue*

To 1.5-ml aliquots withdrawn at various stages of modification (containing 0.25 μmol of SGPB), 1.5 μmol of *N-tert*-butoxycarbonyl-glycyl-L-leucyl-L-phenylalanine chloromethyl ketone in 20 μl of dioxane was added. The samples were

incubated at room temperature for 30 min to ensure full inactivation of the residual active enzyme, which would otherwise interfere with determination of the *N*-terminal residue, due to autodigestion through the reaction with KCNO (Gertler & Hofmann, 1967). The determination of the residual, unmaleylated *N*-terminal amino acid residue was then carried out according to Stark (1967), as modified by Gertler & Hofmann (1967), but without preincubation at pH 3.

RESULTS

Determination of the pK of the group that affects SGPB activity at alkaline pH

The activity of the enzyme was measured over the pH range 8.4–11.5, using a constant substrate concentration (Fig. 1). The reaction velocity (v) at each pH was divided by the respective (H^+) and plotted as a function of the v, according to the linear equation: $v/(H^+) = V_{max}K_2' - v/K_2'$.

A linear plot with a highly significant correlation coefficient of 0.79 ($n = 136$) was obtained using a least-squares program, indicating that the velocity is dependent on ionization of a single group. The apparent ionization constant K_2' was calculated from the slope, resulting in $K_2' = 1.47 \times 10^{-11}$ or $pK_2' = 10.84$, and the V_{max} from the intercept. These values were used for the calculation of the theoretical dissociation curve also shown in Fig. 1. It should be noted that no significant differences were found between the three buffer systems used. Similar analyses performed separately for each of them yielded almost identical results.

Maleylation of SGPB

In a typical experiment, maleylation of 2.0 ml of enzyme solution with 80 µl of 4 M maleic anhydride (added in 16 portions over a period of 60-80 min) resulted in almost complete loss of enzymatic activity, while in a control experiment without maleic anhydride, the activity was not affected. Moreover, the loss of activity was irreversible, persisting for at least a few days in fully or partially modified enzyme at pH 9 and 4°.

The correlation between the loss of activity and the amount of maleylated lysine is presented in Fig. 2. Unfortunately, a ninhydrin-reactive compound that probably does not react with 2,4-dinitrofluorobenzene co-chromatographs with lysine. Therefore, even without maleylation, a small peak corresponding to 0.4–0.5 residues per molecule is obtained after treatment with 2,4-dinitrofluorobenzene. Further difficulty in estimation of the maleylated lysine results from the fact that the lysine is only one out of the 184 amino acids of SGPB. Therefore, in order to minimize the experimental error, over 20 determinations at various stages of modification were performed. In spite of these experimental difficulties, a highly significant positive correlation (correlation coefficient = 0.82, $P < 0.01$) between the modification of 0.9 lysine residue per molecule of SGPB and the loss of enzymatic activity was obtained.

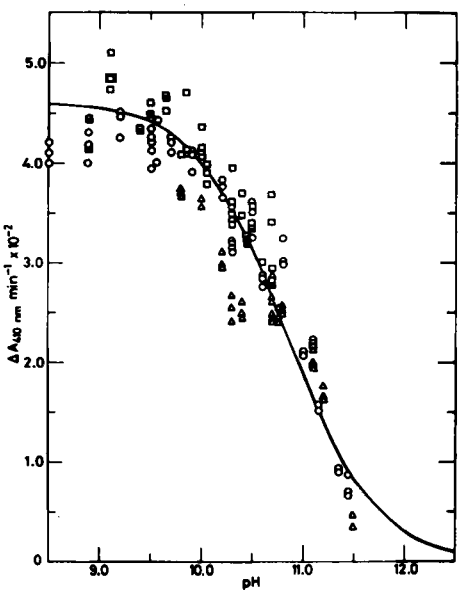

FIGURE 1

Activity of SGPB at alkaline pH: (□) glycine-NaOH buffer, (△) salicylate-NaOH-glycine buffer, (○) universal buffer. The solid line represents the theoretical curve for $pK_2' = 10.84$ and $V_{max} = 0.046 \triangle A_{410\,nm}$/min/10 µg SGPB (1.0 ml reaction volume).

On the other hand, as shown in Table 1, the loss of activity could not be correlated with the maleylation of the α-amino group of the *N*-terminal isoleucine. For instance, when maleylation of this group reached 61% or 82.9% completion, the corresponding loss of

enzymatic activity was only 22.7% and 39.8%, respectively.

Although presented results cannot prove beyond any doubt that the maleylation of the α-amino group does not affect the activity at all, in view of the former report (Siegel & Awad, 1973) and the tertiary structural analysis (Delbaere *et al.*, 1975) it seems rather improbable.

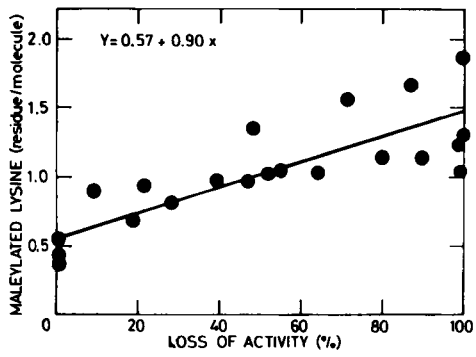

FIGURE 2

Effect of maleylation of the ε-amino group of lysine on SGPB activity. The solid line represents the linear regression calculated from 21 experiments.

DISCUSSION

Although the overall primary structural homology between SGPB and mammalian pancreatic serine proteases is rather low (less than 20% identical residues), in certain regions known to be important for enzymatic activity, such as the histidine-57, aspartate-102, and serine-195 loops (the numbering is that of chymotrypsinogen) the identity is almost complete (Jurasek *et al.*, 1974). Moreover, tertiary structure analysis revealed basically similar chain conformations, thus suggesting that both types of enzyme are products of divergent evolution (Delbaere *et al.*, 1975). In view of this similarity, it is rather unexpected that one of the characteristic structural features of mammalian serine proteases, namely the electrostatic interaction between the *N*-terminal α-amino group and the side chain carboxyl of aspartate-194, is missing in SGPB (Delbaere *et al.*, 1975). This link, which is formed as a result of zymogen activation, stabilizes the active conformation of the enzyme. Therefore, the ionization of the amino group affects the enzymatic activity at alkaline pH (for review see Hess, 1971). In SGPB, this stabilization is achieved by the electrostatic interaction between aspartate-194 and arginine-169 (Delbaere *et al.*, 1975), which is very unlikely to affect enzymatic activity at alkaline pH. The ionization of the *N*-terminal amino group also does not affect enzymatic activity, since it is located on the surface of the molecule, likely forming an electrostatic bond with aspartate-126 (Delbaere *et al.*, 1975). Its acetylation (Siegel & Awad, 1973) or maleylation (the present work) cannot be correlated to the loss of enzymatic activity.

TABLE 1

Effect of maleylation of the α-amino group of the N-terminal isoleucine on SGPB activity

Sample no.[a]	*N*-terminal isoleucine		Residual activity
	mol/mol	%	%
1	0.82	100.0	100.0
2	0.32	39.0	77.3
3	0.14	17.1	60.2
4	0.11	13.4	36.3
5	0.07	8.5	7.8

[a] Sample no. 1 is an average of two determinations, prior to any addition of maleic anhydride. Samples 2–5 were withdrawn at various stages of modification.

Since the present experiments indicate that enzymatic activity is dependent on a group with $pK_2 = 10.84$, our attention focused on other groups, particularly on lysine-125, which forms an electrostatic bond with the α-carboxyl group of the terminal tyrosine-242. As shown above, maleylation of the ε-amino group, which undoubtedly results in disruption of this link, is directly correlated with the loss of enzymatic activity, indicating that its integrity not only stabilizes the enzyme to denaturants, as suggested before (Delbaere *et al.*, 1975), but also is crucial to the maintenance of the catalytic function.

It should be remembered that SGPB consists of two hydrophobic cores with the catalytic site located at their junction. Therefore we would suggest that the electrostatic interaction between the side chain of the lysine-125 which is located in the *N*-terminal core, and the

α-carboxyl group of the *C*-terminal tyrosine is important factor in the proper alignment of the two cores. Its disruption changes the geometry of the catalytic site, leading to the total loss of enzymatic activity.

Siegel & Awad (1973) observed that acetylation of lysine-125 did not result in loss of activity. It should, however, be remembered that this modification is much less drastic and as proposed by the same authors may result in a conversion of an electrostatic interaction into a hydrogen bond.

The apparent pK value for the hydrolysis of $SucGly_2PheNan$ by SGPB was over 1.3 pH units higher than that for the inhibition of the enzyme by peptide chloromethyl ketone (Gertler, 1974), thus raising the question whether ionization of the same group, most likely lysine-125, affects both reactions, or whether the lower pK obtained in inhibition studies is affected by instability of chloromethyl ketones in high pH. No clear answer can be given at present. However, since in other cases (Hirohara *et al.*, 1977) it has been shown that the pK's governing the k_2 and K_s values may be shifted by the binding of different substrates, the possibility that the same group is involved in both cases can not be excluded. It should be also remembered that the velocity of the reaction was estimated at undersaturating substrate concentration and actually represents k_{cat}/K_m value.

No decrease in k_{cat} for SGPB between pH 8 and 10.8 was recently reported by Bauer (1977), who claimed that pK_2' must be higher than 10.8. Since no studies have been done above this pH no explanation for this discrepancy can be provided at present, although it may result from slight differences in experimental conditions, like presence of organic solvent and $CaCl_2$.

ACKNOWLEDGEMENTS

We wish to thank Dr. S. Blumberg from the Weizmann Institute in Rehovot for the colorimetric substrate, Dr. Kurachi from the University of Washington in Seattle for the peptide chloromethyl ketone, and Dr. W. Fenton from Yale University in New Haven, Connecticut for reading this manuscript.

REFERENCES

Awad, W.M., Jr., Soto, A.R., Siegel, S., Skiba, W.E, Bernstrom, G.G. & Ochoa, M.S. (1972) *J. Biol. Chem.* **247**, 4144–4154

Bauer, C.A. (1977) *Acta Chem. Scand.* **31B**, 637–639

Butler, P.T.G., Harris, J.I., Hartley, B.S. & Leberman, R. (1969) *Biochem. J.* **112**, 679–689

Delbaere, L.T.D., Hutcheon, W.L.B., James, M.N.G. & Thiessen, W.E. (1975) *Nature* **257**, 756–758

Gertler, A. (1974) *FEBS Letters.* **43**, 81–85

Gertler, A. & Hofmann, T. (1967) *J. Biol. Chem.* **242**, 2522–2527

Gertler, A. & Trop, M. (1971) *European J. Biochem.* **19**, 90–96

Gertler, A., James, M.N.G., Brayer, G.D. & Delbaere, L.T.D. (1977) *Israel J. Med. Sci.* **13**, 929–930

Hess, G.P. (1971) in *The Enzymes*, 3rd edn. (Boyer, P., ed.) vol. 3, pp. 213–249, Academic Press, New York and London

Hirohara, H., Phillip, M. & Bender, M.L. (1977) *Biochemistry* **16**, 1573–1580

Jurasek, L., Johnson, P., Olafson, R.W. & Smillie, L.B. (1971) *Can. J. Biochem.* **49**, 1195–1201

Jurasek, L., Johnson, P., Olafson, R.W., Smillie, L.B., Gertler, A., Levy, S. & Ericsson, L.H. (1974) *Biochem. Biophys. Res. Commun.* **61**, 1095–1100

McKenzie, H.A. (1969) in *Data for Biochemical Research*, 2nd edn. (Dawsin, R.M.C., Elliot, D.C., Elliot, W.H. & Jones, K.M., eds.), pp. 476–508, Oxford University Press, New York and Oxford

Narahashi, Y. & Fukumaka, J. (1969) *J. Biochem.* **66**, 743–745

Narahashi, Y. & Yoda, K. (1973) *J. Biochem.* **73**, 831–841

Siegel, S. & Awad, W.M. (1973) *J. Biol. Chem.* **248**, 3233–3240

Stark, G.R. (1967) *Methods Enzymol.* **11**, 125–138

Trop, M. & Birk, Y. (1970) *Biochem. J.* **116**, 19–25

Wahlby, S. (1968) *Biochim. Biophys. Acta* **151**, 394–401

Address:

Hebrew University of Jerusalem
Faculty of Agriculture
Department of Agricultural Biochemistry
P.O. Box 12
Rehovot
Israel